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## INTRODUCTION:

The development of human tumors is the result of multiple events including the loss of tumor suppressor genes and the activation of oncogenes. Together these events undermine the signals involved in normal growth control. This proposal has been designed to examine the *in vivo* function of the *mdm2* oncogene in concert with the tumor suppressor *p53*. In addition, the interaction of MDM2 with other proteins and their effect on MDM2 function and tumor development will be examined.

The *mdm2* gene was originally cloned from a transformed murine cell line (3T3DM) as one of three amplified genes stably maintained in the form of double minutes (1). The overexpression of *mdm2* alone in primary rat embryo fibroblasts (REFs) resulted in immortalization or in transformation when transfected with the activated *ras* gene leading to the hypothesis that *mdm2* functioned as an oncogene in the process of cell transformation (2).

Subsequently, MDM2 was discovered as a protein that bound the *p53* tumor suppressor (3). Moreover, the binding of MDM2 to *p53* inactivated *p53* function as a transcriptional activator. The interaction of MDM2 with *p53* created much excitement in the field since the *p53* tumor suppressor gene is mutated in greater than 50% of human cancers (4). Cloning and localization of the human *mdm2* gene indicated that it resides on human chromosome 12q13-14, a region often altered in sarcomas (5). Analysis of both osteosarcomas and soft tissue sarcomas revealed amplification of the *mdm2* gene in approximately one third of tumors (5, 6). Additional experiments showed amplification or overexpression of *mdm2* in glioblastomas and breast carcinomas (7-9). These data thus led to the hypothesis that overexpression of MDM2 by gene amplification represents an alternate mechanism of inactivating *p53* function.

To examine the interaction of *p53* and MDM2 *in vivo*, we deleted the *mdm2* gene in mice using homologous recombination in ES cells (10). *mdm2* null mice died during embryogenesis between 4.5 and 6.5 days of gestation. Since one of the functions of MDM2 is the negative regulation of *p53*, we hypothesized that the embryonic lethality seen in *mdm2* homozygous mutants was due to an inability to down-regulate *p53* function. *p53* null mice are viable (11, 12) and we therefore tested our hypothesis by interbreeding mice heterozygous for both *mdm2* and *p53* genes. Strikingly, we found viable mice that were homozygous null for both *mdm2* and *p53*. Rescue of the *mdm2*  $-/-$  lethality in a *p53* null background suggests that a critical *in vivo* function of MDM2 is the negative regulation of *p53* activity.

However, evidence has accumulated indicating that MDM2 has separate functions in addition to its interaction and inhibition of *p53* function. In some sarcomas, both amplification of *mdm2* and mutations in *p53* were found (13). Patients with these tumors had significantly reduced survival as compared to those

with only one of these alterations. These data are indicative of independent functions. Indeed, recent developments suggest that MDM2 binds other factors implicated in growth control. MDM2 binds another tumor suppressor, the retinoblastoma (Rb) gene product (14). This interaction also disrupts Rb function as a growth suppressor and inhibitor of transcription. In addition, a complex was detected between MDM2 and E2F1, a transcription factor important for the G1/S transition (15). In this case, however, the interaction further stimulated the activity of E2F1. Thus, MDM2 appears to inhibit Rb and p53 function, but its interaction with E2F1 suggests that in addition to inactivation of tumor suppressors, MDM2 can augment cell proliferation by activating genes involved in S-phase progression.

Our hypothesis is that MDM2 is an important component of the p53 pathway, but it has additional independent functions that affect tumorigenesis.

The specific aims of this proposal are:

- 1) to determine the mechanism of death of the *mdm2*<sup>-/-</sup> embryo by investigating growth arrest and apoptosis
- 2) to examine *mdm2*<sup>-/-</sup> *p53*<sup>-/-</sup> mice for timing and spectrum of tumor development
- 3) to assay for other *in vivo* interactions by mating *mdm2*<sup>+/-</sup> mice with *p21* null mice and with *Rb*<sup>+/-</sup> mice
- 4) to analyze MDM2 function using cell lines developed from the *mdm2/p53* null mice.

#### BODY:

Experimental methods, assumptions, and procedures:

*Generation of Transgenic mice.* The ovine  $\beta$ -lactoglobulin promoter expression vector was a gift from A.J. Clark (Edinburgh Research Station, Scotland, UK). An *mdm2* minigene bearing two introns was constructed by replacement of the *AatII-EcoO109I* cDNA fragment with the corresponding fragment (1400bp) from the *mdm2* minigene containing introns 7 and 8. An *EcoRV* fragment containing the *mdm2* minigene was subcloned into the *EcoRV* site of the  $\beta$ -lactoglobulin expression vector. Digestion with *SalI* and *XbaI* released the 9.7-kb transgene from the vector. The fragment was purified on a 0.8% low melting agarose gel by cutting out the transgenic fragment and digesting with GELase (Epicentre). The DNA was then purified by Resource Q anion exchange FPLC (Pharmacia) and ethanol precipitated. The fragment was resuspended in 10 mM Tris (pH 7.4), 0.1 mM EDTA at a final concentration of 3 ng/ $\mu$ l. Microinjection of B6C3F2 zygotes was performed. Mice with the p53 null allele were obtained from Tyler Jacks and backcrossed onto C57Bl/6J.

*PCR analysis and Southern blotting.* DNA was isolated from 1-cm tail biopsies and subjected to PCR analysis. Two different primer sets were used to identify transgenic animals. One set spans the BLG promoter/MDM2 transgene junction. The forward primer is in the BLG promoter (BLG 5'-CCTGTCCTTGTCTAAGAGGCTGAC), and the reverse primer is in the *mdm2* gene (MDM2 5'-CGAAGCTGGAATCTGTGAGGTG) and yields a PCR fragment of 590 bases. The second set of primers amplifies a fragment from the BLG promoter (F2 5'-CCGTCCTGGGGTTATTATGACTC; B2 5'-TCCAAACCATGTGACCGTGG) and yields a predicted fragment of 200 bases. In genotyping the p53<sup>-/-</sup> animals, primers X6.5 (5'-ACAGCGTGGTGGTACCTTAT) and X7 (5'-TATACTCAGAGCCGGCCT) gave a 375-bp band for the wild-type allele and MCI (5'-ATCAGGACATAGCGTTGGCTACCC) and X7 gave a 525-bp band for the p53<sup>-/-</sup> allele (Jacks et al. 1994). Both bands were visible in p53<sup>+/-</sup> animals. PCR assay conditions were 10 mM Tris-HCl (pH8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 10  $\mu$ M deoxynucleotide triphosphate mixture, 0.65 ng/ $\mu$ l primers, 3  $\mu$ l 1:100 dilution of tail DNA, and 0.5 units native Taq DNA polymerase (Perkin Elmer-Cetus) in a 50- $\mu$ l reaction mixture. Forty cycles of denaturation (1 min, 94°C), annealing (1 min, 52°C) and extension (2 min, 72°C) were performed in a Techne PHC-3 thermal cycler. Founder animals that were positive in the PCR assay were confirmed by Southern blotting analysis. Approximately 10  $\mu$ g of genomic tail DNA was digested with *Bam*HI and electrophoresed on a 0.8% agarose gel. The DNA was transferred to a nitrocellulose membrane using standard procedures and hybridized with a radiolabeled 1.5-kb *Bam*HI/*Sph*I fragment from the BLG promoter, yielding a band at 2.2 kb that is specific for the transgene.

*Preparation and analysis of RNA.* Total cellular RNA was prepared from mouse tissues using TRI REAGENT<sup>®</sup> (Molecular Research Center, Inc.) according to the manufacturer's instructions. RNA expression was analyzed in a T2 RNase protection assay. To make the MDM2 antisense probe the *Bam*HI fragment of the *mdm2* minigene was subcloned into Bluescript KS<sup>+</sup> (Stratagene). The plasmid was linearized at the *Sa*I site and used as a template for *in vitro* transcription with T7 RNA polymerase (Boehringer Mannheim). For an internal control, a 300-nucleotide antisense riboprobe for the  $\beta$ -actin gene (Ambion) was synthesized. Twenty-five to fifty micrograms of total cellular RNA was combined with  $5 \times 10^5$  -  $1 \times 10^6$  cpm of synthesized probe and ethanol precipitated and dried. The pellet was resuspended in 40  $\mu$ l of hybridization buffer containing 80% formamide, 40 mM 1,4 piperazinediethanesulfonic acid (PIPES, pH 6.7), 0.4 M NaCl, and 1 mM EDTA and incubated overnight at 60°C. Three hundred and fifty microliters of unlabeled T2 RNase digestion buffer [50 mM sodium acetate (pH 4.5), 2 mM EDTA, 100 mM NaCl] containing 20 units/ml T2 RNase were added to each sample and incubated at 33°C for 1h. Samples were ethanol precipitated, resuspended in 8  $\mu$ l of gel loading buffer (Ambion), subjected to electrophoresis through a 5% polyacrylamide gel, and analyzed by autoradiography.



*Histology and Immunohistochemistry.* Tissues obtained from animal necropsies or mammary gland biopsies were fixed in Z-fix neutral buffered formalin (Anatech) or Bouin's fixative (Sigma). Five-micrometers thick paraffin sections were stained with hematoxylin and eosin for histological analysis. Mice received an intraperitoneal injection of BrdU (Boehringer-Mannheim) at 120 mg per kg body weight 1-1.5h before sacrifice to label cells in S-phase (Kim et al. 1993). For BrdU, PCNA, and MDM2 staining, 5- $\mu$ m thick sections were placed on ProbeOn<sup>+</sup> slides (Fisher Scientific) to insure adhesion during processing. Tissue sections were incubated with a monoclonal antibody to BrdU (1:500, Accurate Chemical and Scientific Corporation) or PCNA (1:500, Dako Corporation). Rabbit polyclonal antiserum specific for the MDM2 protein was a generous gift from Arnold Levine (Princeton University). The MDM2 antiserum was diluted 1:250 for immunocytochemistry. The primary antibodies were detected using the Vectastain ABC immunoperoxidase system with 3,3'-diaminobenzidine tetrahydrochloride (DAB) as the chromagen substrate. In addition, the DAB enhancer (Zymed) was used for MDM2 detection. Positive staining for PCNA was determined based on cellular distribution and intensity of the brown to black staining, which correlates with the different phases of the cells cycle as previously reported. For whole-mount analysis, the first abdominal (4th) mammary gland was dissected and stretched on a glass slide. After air drying, the gland was defatted and fixed overnight in acetone. The glands were then stained in Harris' hematoxylin overnight, destained in a solution of 2% HCl in 70% ethanol, and then dehydrated in a graded ethanol series. The dehydrated tissue was cleared overnight in toluene and mounted.

*Ploidy Analysis.* Paraffin sections were cut 6  $\mu$ m thick and placed on Probe On<sup>+</sup> slides. Tissue sections were stained using the Feulgen staining kit (Becton Dickinson Cellular Imaging systems). An unstained calibration slide and an unstained mammary sample were included as method controls in the staining procedure. Nuclei in sections from nontransgenic control mammary epithelial cells were used to establish the 2N DNA content using DNA Image Cytometry (Roche Pathology WorkStation, Roche Image Analysis Systems, Inc, now LabCorp). The optical density of nuclei from transgenic mice was quantitated relative to the control nuclei. The OD of approximately 100 nuclei per sample was determined, and the results were graphed. The prominent histogram peaks were labeled to give an overall DNA index for each peak (LabCorp, Research Triangle Park, NC).

*Western blot analysis.* At necropsy, tissues were frozen at -70°C. For Western analysis, lysates of tissues were made in lysis buffer (50 mM Tris pH 7.4, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate) containing protease inhibitors by dounce homogenization followed by sonication. For an MDM2 control, 3T3 DM cell (1) pellets were lysed in 50 mM Tris pH 8, 5 mM EDTA, 150 mM NaCl, and 0.5% NP40 containing protease inhibitors. After centrifugation, a protein assay (Biorad) was used to determine the protein concentration of lysates. Fifty micrograms of protein lysate was loaded on



a 4-12% NuPAGE gradient gel (Novex) and electrophoresed at 200 V. Proteins were transferred to nitrocellulose using the NuPAGE (Novex) transfer system according to the manufacturer's instructions. Blots were blocked with 2.5% milk in TBS-T for 1h and then incubated with an MDM2-specific polyclonal antiserum at 1:500 dilution for 1h at room temperature. After being washed in five changes of TBS-T, the blot was incubated with a Donkey anti-rabbit antibody (Amersham) at a 1:4000 dilution for 1h at room temperature. The MDM2 protein was detected using the ECL detection system (Amersham).

## Results and discussion:

The early embryonic lethality seen in *mdm2* null embryos is due to an inability to inactivate p53, and is completely rescued by the absence of p53 (10, 16). Because the MDM2/p53 interaction is critical to the survival of this *in vivo* model, it is ideal for studying the p53 pathway, specifically the contribution of downstream effectors of p53. One of the genes activated by p53 is *p21* (17). *p21* is the only known p53 target that binds and inhibits the function of the cyclin-cyclin dependent kinase complexes and thus halts cell cycle progression (18, 19).

To examine the importance of *p21* as an effector of p53 function *in vivo*, we asked whether deletion of *p21* could substitute for deletion of *p53* in the rescue of *mdm2*<sup>-/-</sup> embryos. Mice heterozygous for *mdm2* and null for *p21* (20) were mated with each other. In this cross, 25% of the mice are expected to be double null. Of 46 mice born, 11 (24%) were *mdm2*<sup>+/+</sup>*p21*<sup>-/-</sup>, 35 (76%) were *mdm2*<sup>+/+</sup>*p21*<sup>-/-</sup>, and none were *mdm2*<sup>-/-</sup>*p21*<sup>-/-</sup>. These data indicate that the loss of *p21*, unlike the loss of *p53*, does not rescue the lethality of *mdm2*<sup>-/-</sup> embryos.

The possibility remained that loss of *p21* was insufficient to allow *mdm2*<sup>-/-</sup> embryos to survive until birth, but that a partial rescue occurred. Again *mdm2*<sup>+/+</sup>*p21*<sup>-/-</sup> mice were mated with each other, and pregnant females were sacrificed during gestation. Genotyping of embryos at days 9.5 and 7.5 of gestation did not yield any double null embryos. To address the possibility that rescue of *mdm2*<sup>-/-</sup> lethality at 5.5 days of development by loss of *p21* delayed death by a very short period of time, the deciduae containing embryos from a cross between *p21*<sup>-/-</sup>*mdm2*<sup>+/+</sup> mice were dissected at 5.5 days of gestation and paraffin embedded. Of twenty five deciduae sectioned, five (20%) showed lack of embryo development reminiscent of the empty deciduae seen in *mdm2* heterozygous crosses (10). Thus, loss of *p21*, in contrast to *p53* deletion, did not rescue even partially the lethality of *mdm2*<sup>-/-</sup> embryos.

We further examined the ability of p53 to activate transcription in this system. We analyzed induction of p53 protein, and *p21* and *mdm2* mRNAs in embryos at 5.5 days of development after irradiation with 5.0 Gy. In embryos, p53 was induced

by 100 minutes with no induction of *p21* mRNA (see appendix A). In contrast, another transcriptional target of p53, the *mdm2* gene (21-24), was induced by gamma-radiation in both the embryo and surrounding maternal tissue of the deciduae. Anti-p21 and -MDM2 antibodies were used to detect protein. The p21 antibody did not detect protein, while MDM2 was clearly produced in these embryos. As controls, the p21 antibody was able to detect p21 in uterine muscle, but not in the same tissue from *p21* null mice.

While the lethality of *mdm2* null embryos is rescued in the absence of p53, surprisingly, the loss of *p21* could not substitute, even partially, for loss of p53. p53 is a regulator of multiple pathways and the possibility exists that no single target can effect the functions of p53, a fact that is supported by the lack of mutations in *p21* in human tumors (24-26) and the lack of susceptibility of *p21* null mice to tumorigenesis (20, 27). However, the lack of *p21* induction by p53 in wild type embryos at 5.5 days of development indicates that p21 is not part of this pathway. Thus, we have unveiled an *in vivo* situation in which the ability of p53 to exert its effects is not dependent on activation of *p21*.

To determine the role of MDM2 in the cell cycle and in tumorigenesis and whether it has a function independent of p53, an MDM2 minigene was expressed during gestation and lactation in the mammary gland of both wild-type p53 (*p53*<sup>+/+</sup>) and p53 knockout (*p53*<sup>-/-</sup>) mice using the ovine  $\beta$ -lactoglobulin promoter (see appendix B). In six different transgenic mouse lines, deregulated expression of MDM2 inhibited normal development and morphogenesis of the mammary gland, and caused hypertrophy and nuclear abnormalities. These abnormalities included both multinucleated cells and enlarged cells with giant nuclei. Although there were fewer epithelial cells present in the transgenic mammary gland, no apoptosis was observed. Instead, BrdU incorporation and PCNA staining showed that 12-27% of the transgenic mammary epithelial cells were in S-phase at a time when normal cells were terminally differentiated. Analysis of DNA content showed that 30-45% of the cells were polyploid, with DNA contents up to 16N, indicating that overexpression of MDM2 caused mammary epithelial cells to undergo multiple rounds of S-phase without cell division. This phenotype was similar in the *p53*<sup>+/+</sup> and *p53*<sup>-/-</sup> background, demonstrating a role for MDM2 in the regulation of DNA synthesis that is independent of the ability of MDM2 to inhibit p53 activity. Additionally, multiple lines of BLGMDM2 transgenic mice developed mammary tumors, confirming that overproduction of MDM2 contributes to tumorigenesis in epithelial cells *in vivo*.

## Recommendations:

Most of aim 3 is complete. The genetic interaction with p21 has been published. In addition, aim 2 is almost complete and ready for submission. We will also be working on the *mdm2* transgenic mouse model since it led to the development of breast tumors. This is a beautiful model to determine critical MDM2 interactions in tumor development.

## CONCLUSIONS:

The work presented in this progress report makes major contributions to our understanding of p53 and MDM2. In the first study, we learned that the loss of p21 cannot substitute, even partially, for loss of p53 in an *in vivo* model dependent on loss of p53 for survival. Thus, it appears that no single target of p53 can effect its functions.

Another *in vivo* model was used to study the functions of MDM2. The overexpression of *mdm2* in the breast epithelium led to multiple rounds of S-phase without cytokinesis. This phenotype was independent of p53. Moreover the overexpression of *mdm2* led to breast carcinomas and adenocanthomas.

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Wen Yang, Ph.D.

## Deletion of *p21* cannot substitute for *p53* loss in rescue of *mdm2* null lethality

The MDM2 protein negatively regulates the activity of the p53 tumour suppressor by binding its transcriptional activation domain<sup>1,2</sup>. In fact, the early embryonic lethality seen in *mdm2* null embryos is due to an inability to inactivate p53, and is completely rescued by the absence of p53 (refs 3,4). Because the MDM2/p53 interaction is critical to the survival in this *in vivo* model, it is ideal for studying the p53 pathway, specifically the contribution of downstream effectors of p53. One of the genes activated by p53 is *p21* (ref. 5). *p21* is the only known p53 target that binds and inhibits the function of the cyclin-cyclin-dependent kinase complexes and thus halts cell-cycle progression<sup>6,7</sup>.

To determine the importance of the p53 target, *p21*, in rescue of the *mdm2*<sup>-/-</sup> lethality, we first asked whether these genes were expressed in early development.

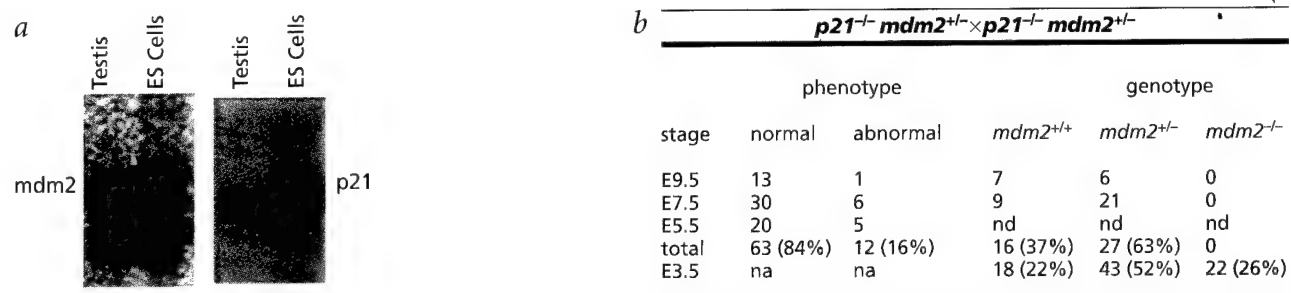
Northern analysis using RNA from embryonic stem cells obtained from 3.5-day blastocysts revealed that *p53* and its effectors, *mdm2* and *p21*, were highly expressed in these cells (Fig. 1a). Thus, these components of the p53 pathway were present early in development.

To examine *p21* as an effector of p53 function *in vivo*, we asked whether deletion of *p21* could substitute for deletion of *p53* in the rescue of *mdm2*<sup>-/-</sup> embryos. Mice heterozygous for *mdm2* and null for *p21* were mated with each other<sup>3,8</sup>. In this cross, 25% of the mice are expected to be double null. Of 46 mice born, 11 (24%) were *mdm2*<sup>+/-</sup> *p21*<sup>-/-</sup>, 35 (76%) were *mdm2*<sup>+/-</sup> *p21*<sup>-/-</sup> and none were *mdm2*<sup>-/-</sup> *p21*<sup>-/-</sup>. These data indicate that the loss of *p21*, unlike the loss of *p53*, does not rescue the lethality of *mdm2*<sup>-/-</sup> embryos.

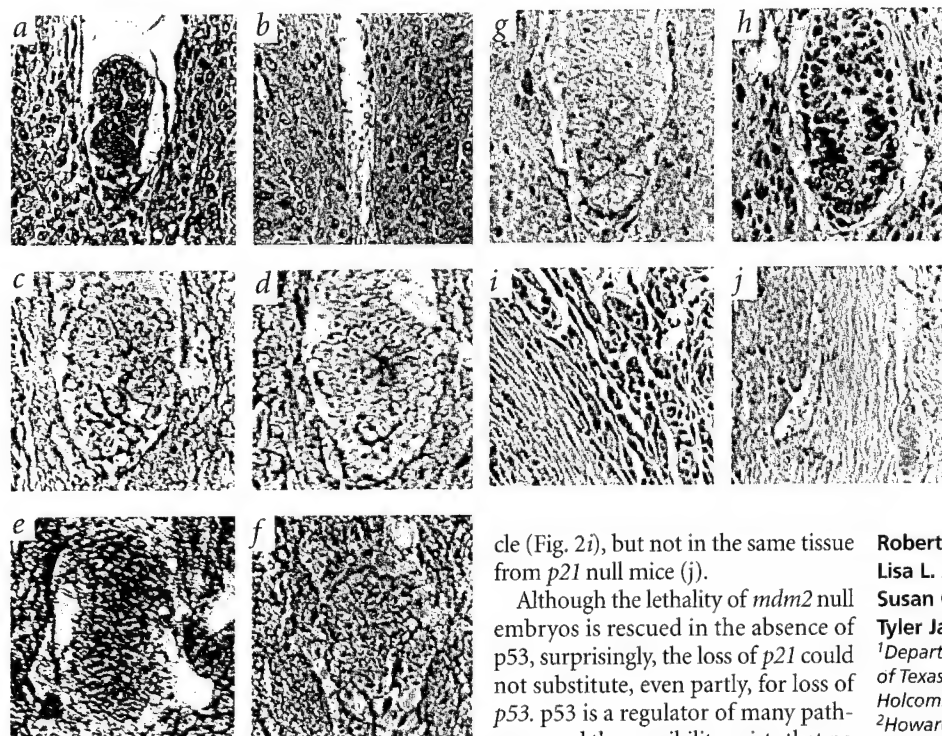
The possibility remained that loss of *p21*

was insufficient to allow *mdm2*<sup>-/-</sup> embryos to survive until birth, but that a partial rescue occurred. Again, *mdm2*<sup>+/-</sup> *p21*<sup>-/-</sup> mice were mated with each other, and pregnant females were examined during gestation. Genotyping of embryos at days 9.5 and 7.5 of gestation did not yield any double null embryos (Fig. 1b). To address the possibility that rescue of *mdm2*<sup>-/-</sup> lethality at 5.5 days of development by loss of *p21* delayed death by a very short time, the deciduae containing embryos from a cross between *p21*<sup>-/-</sup> *mdm2*<sup>+/-</sup> mice were dissected at 5.5 days of gestation and paraffin embedded. Of 25 deciduae sectioned, five (20%) showed lack of embryo development reminiscent of the empty deciduae seen in *mdm2* heterozygous crosses<sup>3</sup> (Fig. 2a,b). Thus, loss of *p21*, in contrast to *p53* deletion, did not even partly rescue the lethality of *mdm2*<sup>-/-</sup> embryos.





**Fig. 1 a**, RNA isolated from testes and ES cells was hybridized with the *mdm2*, *p53* and *p21* probes. The ethidium-stained RNA gel is shown (bottom panel) to indicate appropriate loading. **b**, Genetic analysis of *p21*/*mdm2* embryos. Only the *mdm2* genotype is shown, as all mice from this cross were *p21* null. The abnormality seen in this cross is an empty decidua. na, not applicable; nd, not determined.



**Fig. 2** The phenotype and expression of several genes were assayed in mouse embryos at 5.5 days post coitum. Deciduae from crosses between *p21*<sup>-/-</sup> *mdm2*<sup>+/+</sup> mice were dissected, paraffin embedded and sectioned. While most embryos were normal (**a**), 20% of the embryos had an abnormal phenotype (**b**). To assay for *p21* induction by *p53*, normal embryos were treated with 5.0 Gy of ionizing radiation and hybridized *in situ* with anti-sense (**c**) and sense (**d**) *p21* probes. As a control for induction of the *p53* pathway, *in situ* hybridization with antisense (**e**) and sense (**f**) *mdm2* probes was performed on irradiated embryos. Anti-*p21* (**g**) and anti-*mdm2* antibodies (**h**) were used to detect protein. As controls, anti-*p21* antibodies were also used on uteri of normal (**i**) and *p21* null mice (**j**).

We further examined the ability of *p53* to activate transcription in this system. We analysed induction of *p53* protein, and *p21* and *mdm2* mRNAs in embryos at 5.5 days of development after irradiation with 5.0 Gy. In embryos, *p53* was induced by 100 minutes (data not shown), with no induction of *p21* mRNA (Fig. 2c,d). In contrast, another transcriptional target of *p53*, the *mdm2* gene<sup>9-11</sup>, was induced by gamma radiation in both the embryo and surrounding maternal tissue of the decidua (Fig. 2e,f, respectively). Anti-*p21* and -*mdm2* antibodies were used to detect protein. The *p21* antibody did not detect protein, whereas MDM2 was clearly produced in these embryos (Fig. 2g,h, respectively). As controls, the *p21* antibody was able to detect *p21* in uterine mus-

cle (Fig. 2i), but not in the same tissue from *p21* null mice (j).

Although the lethality of *mdm2* null embryos is rescued in the absence of *p53*, surprisingly, the loss of *p21* could not substitute, even partly, for loss of *p53*. *p53* is a regulator of many pathways, and the possibility exists that no single target can affect the functions of *p53*—a possibility supported by the lack of mutations in *p21* in human tumours<sup>12-14</sup> and the lack of susceptibility of *p21* null mice to tumorigenesis<sup>8,15</sup>. However, the lack of *p21* induction by *p53* in wild-type embryos at 5.5 days of development indicates that *p21* is not part of this pathway. Thus, we have unveiled an *in vivo* situation in which the ability of *p53* to exert its effects is not dependent on activation of *p21*.

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# Targeted expression of MDM2 uncouples S phase from mitosis and inhibits mammary gland development independent of p53

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MDM2 is a cellular protein that binds to and inactivates the p53 tumor suppressor protein. Although *mdm2* has been shown to function as an oncogene in vitro, all studies to date have assessed MDM2 activities in the presence of p53, implicating p53 inactivation in MDM2-directed transformation. To determine the role of MDM2 in the cell cycle and in tumorigenesis and whether or not this role is dependent on p53, an MDM2 minigene was expressed during gestation and lactation in the mammary gland of both wild-type p53 (p53+/+) and p53 knockout (p53-/-) mice using the bovine  $\beta$ -lactoglobulin promoter. In six different transgenic mouse lines, deregulated expression of MDM2 inhibited normal development and morphogenesis of the mammary gland, and caused cellular hypertrophy and nuclear abnormalities. These abnormalities included both multinucleated cells and enlarged cells with giant nuclei. Although there were fewer epithelial cells present in the transgenic mammary gland, no apoptosis was observed. Instead, BrdU incorporation and PCNA staining showed that 12%–27% of the transgenic mammary epithelial cells were in S phase at a time when normal cells were terminally differentiated. Analysis of DNA content showed that 30%–45% of the cells were polyploid, with DNA contents up to 16N, indicating that overexpression of MDM2 caused mammary epithelial cells to undergo multiple rounds of S phase without cell division. This phenotype was similar in the p53+/+ and p53-/- background, demonstrating a role for MDM2 in the regulation of DNA synthesis that is independent of the ability of MDM2 to inhibit p53 activity. Additionally, multiple lines of BLGMDM2 transgenic mice developed mammary tumors, confirming that overproduction of MDM2 contributes to tumorigenesis in epithelial cells in vivo.

[Key Words: *mdm2*; p53; transgenic mice; cell cycle; tumor]

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The MDM2 cellular protein interacts with the p53 tumor suppressor and inhibits p53 function (Momand et al. 1992). Specifically, MDM2 binds to the amino-terminal acidic domain of p53 and inhibits p53 transactivation (Chen et al. 1993; Oliner et al. 1993). In addition, overexpression studies in tissue culture have demonstrated that MDM2 can inhibit wild-type p53-mediated G<sub>1</sub> arrest and apoptosis (Chen et al. 1996; Haupt et al. 1996). The relationship between p53 and MDM2 extends beyond protein-protein interaction. P53 can bind to p53-

responsive elements within the promoter of the *mdm2* gene and activate *mdm2* transcription, thus establishing a negative feedback loop (Barak et al. 1993; Juven et al. 1993; Wu et al. 1993).

Additional clues to the role of the MDM2-p53 interaction have been obtained from deletion of the *mdm2* gene in mice (Jones et al. 1995; Montes de Oca Luna et al. 1995). Mice that are null for MDM2 function are nonviable by day 5 of gestation, whereas mice null for p53 are viable but predisposed to tumors, succumbing to lymphoma and other tumors from 4 to 6 months of age (Donehower et al. 1992; Jacks et al. 1994). Crossing *mdm2* and p53 mutant mice yielded viable progeny that were null for both genes. The rescue of the *mdm2*-/- lethality demonstrated that the ability of MDM2 to inhibit p53 function is critical during early mouse development.

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MDM2 was discovered independent of the interaction with p53, as a gene amplified on double-minute chromosomes in a tumorigenic murine cell line (Cahilly-Snyder et al. 1987; Fakharzadeh et al. 1991). The overexpression of *mdm2* confers a tumorigenic phenotype to NIH-3T3 cells (Fakharzadeh et al. 1991) and cooperates with an activated *ras* gene to transform primary rat embryo fibroblasts (Finlay 1993). In addition, amplification of the *mdm2* gene has been observed in 30%–40% of human sarcomas (Oliner et al. 1992; Ladanyi et al. 1993; Leach et al. 1993; Reifemberger et al. 1993; Cordon-Cardo et al. 1994) and with a low frequency in human carcinomas (Sheikh et al. 1993; Fontana et al. 1994; Quesnel et al. 1994; Marchetti et al. 1995; McCann et al. 1995). The majority of tumors overexpressing *mdm2* retained a wild-type *p53* gene, suggesting that MDM2 can effectively overcome p53 tumor suppressor activity. Some tumors, however, appear to have both mutant p53 and overexpressed MDM2, indicating that both events could contribute to tumor development (Cordon-Cardo et al. 1994).

Recent studies have provided evidence implicating MDM2 as a regulatory protein in the G<sub>1</sub>/S transition period of the cell cycle through interactions with both the E2F1 transcription factor and the retinoblastoma (Rb) tumor suppressor protein in vitro. MDM2 can bind to and stimulate E2F1/DP1 transcriptional activity (Martin et al. 1995), which functions as a positive regulator for entry into S phase. MDM2 also interacts with Rb to inhibit Rb growth regulatory function (Xiao et al. 1995). Taken together, the MDM2 interactions and activities characterized to date suggest that MDM2 plays a role in promoting entry into S phase.

To assess the role of the MDM2 protein in cell cycle regulation and tumorigenesis, and how the interaction between MDM2 and p53 affects these functions, we targeted expression of an MDM2 minigene to the mammary gland in both wild-type and p53<sup>-/-</sup> mice using the ovine milk protein promoter  $\beta$ -lactoglobulin (BLG). The mammary gland is a particularly good model because epithelial cells undergo a normal cycle of proliferation, differentiation, and apoptosis during pregnancy, lactation, and involution following weaning. Promoters that target expression of exogenous genes to the mammary gland have been used to assess the role of genes in cell cycle regulatory pathways, development, and tumorigenesis (Cardiff and Muller 1993). In the mammary gland, tumors have arisen in mice with deregulated expression of *c-neu*, *c-myc*, *Ha-ras*, *Wnt-1*, *int-2*, transforming growth factor- $\alpha$  (TGF- $\alpha$ ), and cyclin D (for review, see Cardiff and Muller 1993; Cardiff and Munn 1995; Amundadottir et al. 1996). In most instances, tumors arise from focal lesions in a time course delayed relative to initial transgene expression, suggesting that secondary events are necessary for tumorigenesis.

The data presented in this report demonstrate that overproduction of MDM2 during pregnancy and lactation results in defective development of the mammary gland. The lack of development observed in the transgenic mammary gland resulted from multiple rounds of

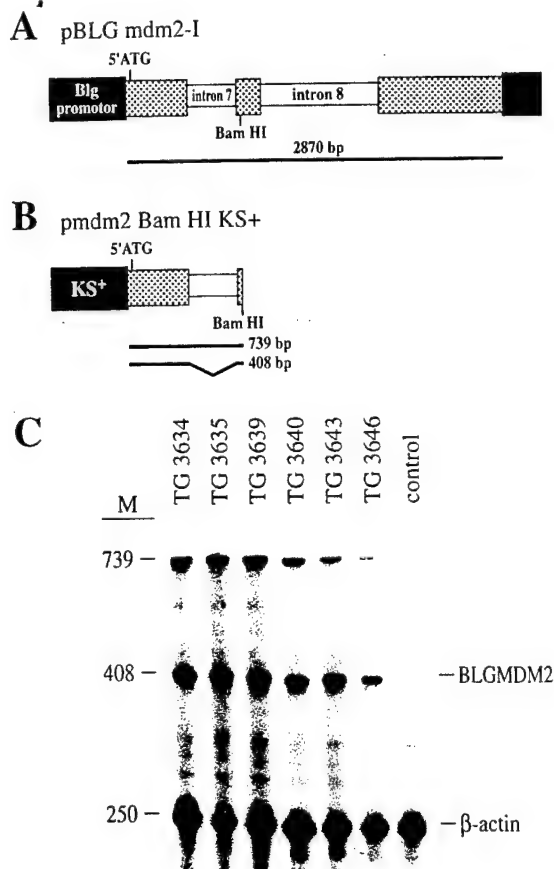
S phase without completion of mitosis. This uncoupling of S phase from mitosis was observed in BLGMDM2 transgenic mice in both the wild-type p53 and the p53<sup>-/-</sup> backgrounds, demonstrating a role for MDM2 that is independent of p53 function. In addition, mammary tumors have developed in mice bearing the BLG-MDM2 transgene from 14 to 18 months of age, demonstrating that deregulated expression of MDM2 can contribute to tumor formation in the mammary gland.

## Results

### *Targeted expression of MDM2 in the murine mammary gland*

To determine the effect of deregulating MDM2 expression in an in vivo system, the ovine BLG promoter was used to direct expression of an *mdm2* minigene to the murine mammary gland. The BLG promoter has been shown to confer tissue-specific, hormonally regulated expression of heterologous genes to the mammary epithelial cells of transgenic mice during pregnancy and lactation (Harris et al. 1991). The construct consists of the *mdm2* cDNA containing the seventh and eighth genomic introns (Montes de Oca Luna et al. 1996) cloned into a BLG vector and is designated BLGMDM2 (Fig. 1A). The transgene was excised from plasmid DNA, and the 9.7-kb linear fragment was microinjected into the pronuclei of B6C3F2 mouse embryos (Osborn et al. 1987). Transgenic founder animals were identified by PCR analysis and confirmed by Southern blotting (data not shown). From these founders, nine independent lines of transgenic mice were established by backcrossing to C57Bl/6J mice.

To assess the expression of the *mdm2* minigene, total RNA was isolated from mid-lactation (day 8–12) mammary glands of transgenic and nontransgenic siblings and subjected to T2 RNase protection analysis. The *Bam*HI fragment of the *mdm2* minigene was subcloned into Bluescript KS<sup>+</sup>, and antisense RNA was synthesized for the probe (Fig. 1B). RNase expression analysis of mid-lactation mammary glands from each of the six highest expressing lines is shown in Figure 1C. The 771-nucleotide antisense *mdm2* probe contained 408 nucleotides of homology to the spliced transgene and 739 nucleotides of homology to the unspliced transgene (cDNA plus genomic intron). Both the spliced and unspliced forms were detected in the RNase protection assay (Fig. 1C). The endogenous murine *mdm2* RNA was detected as a 402-base band in overexposed gels (data not shown). For an internal control, a 300-nucleotide antisense riboprobe containing a 250-nucleotide region of homology to the murine  $\beta$ -actin gene (Ambion) was used. The expression of the BLGMDM2 transgene was 10 to 30 times that of endogenous *mdm2* levels in independent lines. In addition to the mammary gland, expression of the BLG-MDM2 transgene transcript was analyzed using RNA from salivary gland, spleen, kidney, liver, lung, heart, eye/hardian gland, and brain and was not detected in any of these tissues (data not shown). Of the nine origi-



**Figure 1.** Generation of BLGMDM2 transgenic lines. (A) The BLGMDM2 transgene construct contains the BLG promoter (left solid box) and the full-length normal *mdm2* coding sequence (stippled boxes) interrupted by introns 7 and 8 (open boxes) followed by the poly(A) tail from the BLG gene (right solid box). (B) The fragment used to generate the RNA probe for T2 RNase expression analysis protects transgene RNA of 408 bases. (C) T2 RNase expression analysis of mid-lactation mammary glands from the six highest expressing BLGMDM2 transgenic lines.

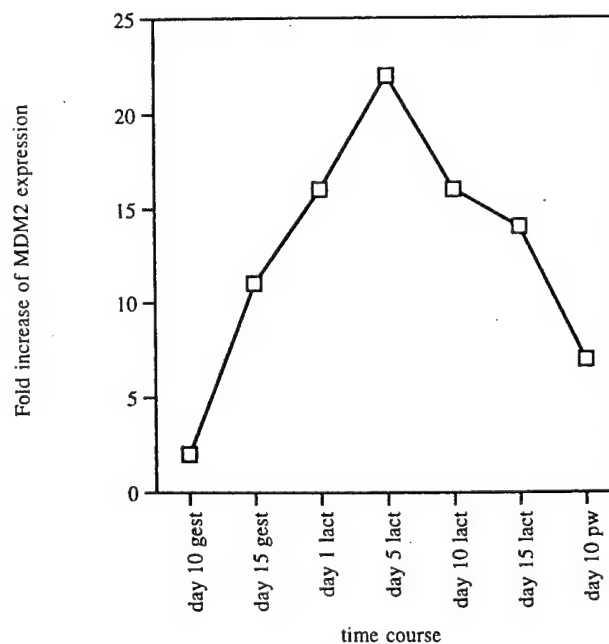
nal lines generated, the six shown expressing the highest levels of MDM2 were analyzed further, two in great detail.

#### Time course of BLGMDM2 expression in the mammary gland during pregnancy and lactation

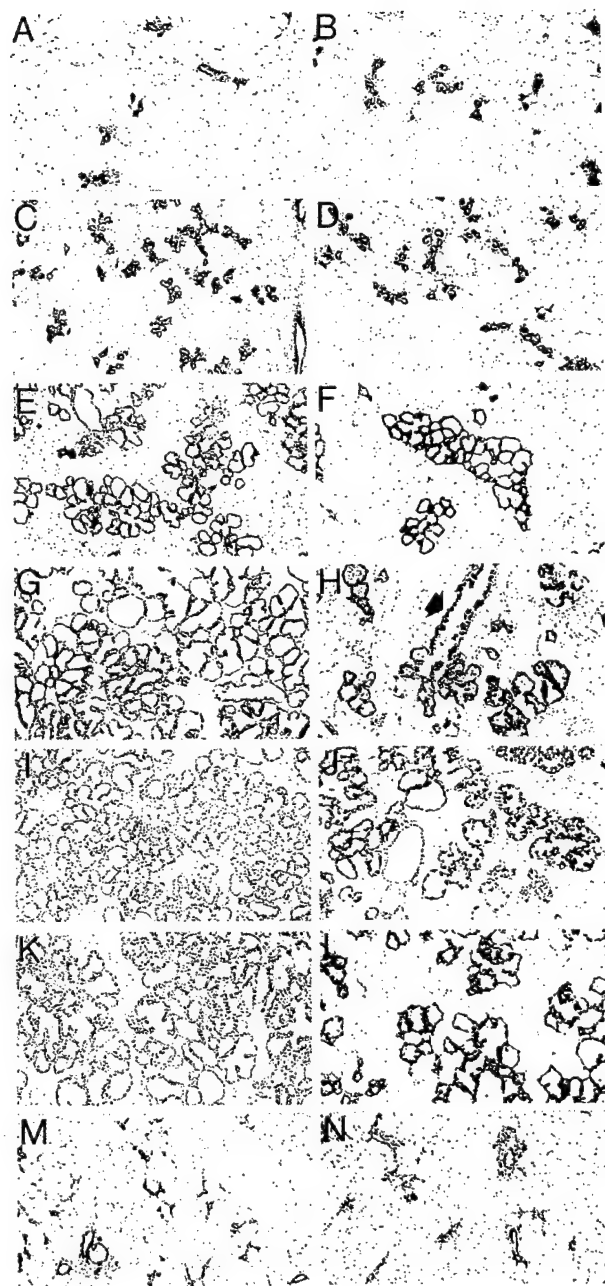
The transgenic line TG3640 displayed both a high level of *mdm2* RNA expression (Fig. 1C) and developmental abnormalities in the mammary gland in preliminary biopsies, and so it was chosen for further analysis. To trace the initiation and development of the abnormalities, mammary glands from mice of line TG3640 were analyzed histologically and for expression of the transgene in a time course experiment. RNA expression (Fig. 2) and hematoxylin and eosin (H&E)-stained sections (Fig. 3) from control and transgenic glands were compared at day 10 and 15 of gestation, day 1, 5, 10, and 15 of lactation,

and day 10 postweaning. Figure 2 shows the quantitation of three different RNase protection experiments that were averaged and graphed over the time course relative to endogenous *mdm2* RNA levels. In the control animals, no transgenic *mdm2* RNA was detected. Transgenic *mdm2* RNA levels were twice the endogenous levels at day 10 of gestation, increased to 12 times the endogenous levels at the end of gestation, and increased to 22 times the endogenous levels by day 5 of lactation. Expression then decreased gradually thereafter.

Concurrent with RNA analysis, a histological examination was performed. The time course of mammary gland development in control animals is shown in Figure 3. In the normal mammary gland, epithelial cells began proliferating during gestation (Fig. 3A,C) until 1 day after parturition, when they form the lobulo-alveolar structures that secrete milk (Fig. 3E, day 1 of lactation). Mammary epithelial cells began to differentiate prior to parturition, and by day 5 of lactation (Fig. 3G), the cells that form the alveoli were terminally differentiated and mitotically quiescent. In comparison, mammary glands from BLGMDM2 transgenic mice did not develop normally. At day 10 (Fig. 3A,B) and 15 of gestation (Fig. 3C,D), control and transgenic glands showed similar growth and development. By day 1 of lactation, however, the transgenic gland (Fig. 3F) appeared to be less devel-



**Figure 2.** Time course of BLGMDM2 transcript expression during pregnancy and lactation. The relative amount of BLGMDM2 (TG3640) RNA expression was calculated and averaged from three separate T2 RNase experiments. The ratio of transgene MDM2 expression to endogenous MDM2 levels in control mice (seen only with overexposure of the gel) was calculated after compensation for actin levels. Transcripts were quantitated with a Molecular Dynamics PhosphorImager. (gest) Gestation; (lact) lactation; (pw) postweaning.



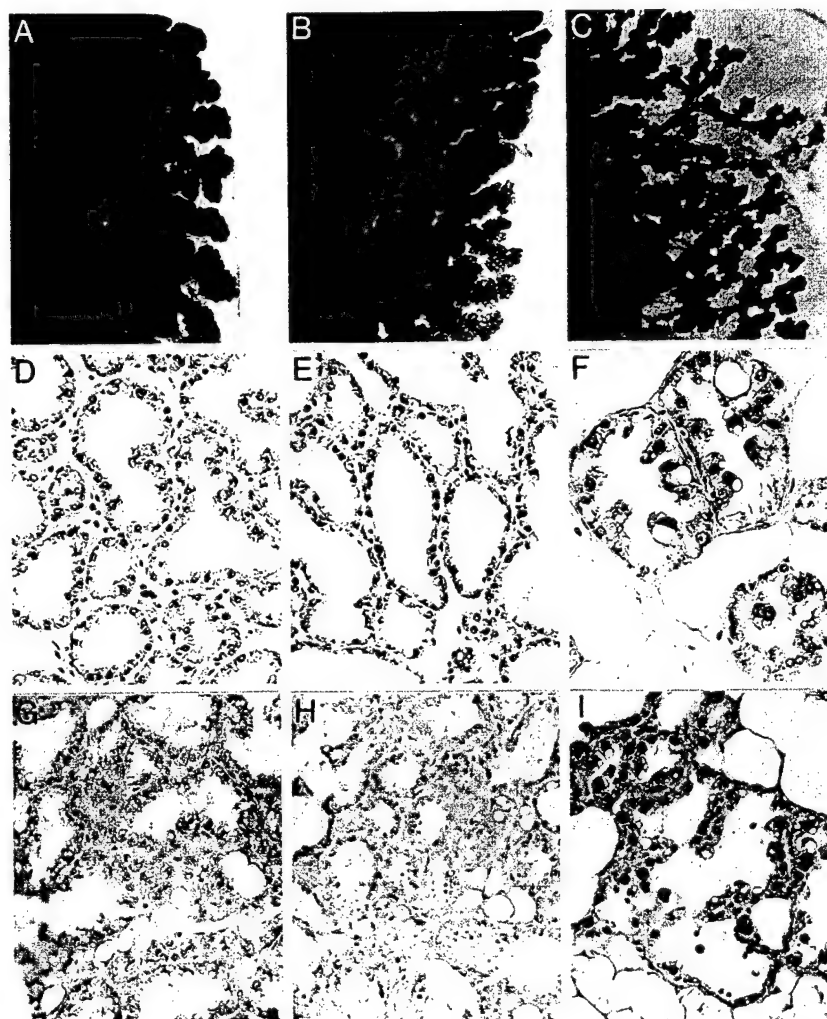
**Figure 3.** Histological analysis of normal and BLGMDM2 (TG3640) mammary glands during pregnancy and lactation. Paraffin sections of mammary tissue taken at various times during the lactation cycle were stained with H&E. Mammary glands from control mice at day 10 of gestation (A), day 15 of gestation (C), day 1 of lactation (E), day 5 of lactation (G), day 10 of lactation (I), day 15 of lactation (K), day 10 of postweaning (M). Mammary glands from BLGMDM2 mice at day 10 of gestation (B), day 15 of gestation (D), day 1 of lactation (F), day 5 of lactation (H; arrow points to ductal hyperplasia visible as a piling up of cells one on top of another), day 10 of lactation (J), day 15 of lactation (L), and day 10 of postweaning (N). (A–N) Magnification, 100 $\times$ .

oped than the control gland (Fig. 3E). On days 5, 10, and 15 of lactation a striking lack of development was apparent in the transgenic glands (Fig. 3H,J,L, respectively) relative to control glands (Fig. 3G,I,K, respectively). The mammary gland of the transgenic mice contained fewer lobulo-alveolar structures and fewer epithelial cells. The epithelial cells lining the basement membrane of the alveoli and ducts had larger nuclei, which stained atypically compared with controls. Ductal hyperplasia was also apparent in the BLGMDM2 transgenics, as shown in the histological section from day 5 of lactation (Fig. 3H, arrow). Whereas normal ducts were lined with a single layer of epithelial cells, the transgenic glands contained cells that piled up along the walls of the ducts. The lack of glandular development resulted in lactation difficulties, so much so that the pups were routinely fostered at birth. Both the control and transgenic glands regressed normally at weaning (Fig. 3M,N). Overall, the appearance of the developmental abnormalities correlated well with the time course of induced transgene expression (Fig. 2).

#### *Comparison of mammary gland development in p53<sup>-/-</sup> and BLGMDM2 transgenic mice*

Because the MDM2 protein can inhibit p53 function (Momand et al. 1992; Chen et al. 1993; Oliner et al. 1993), we asked whether the loss of p53 resulted in a phenotype similar to that of *mdm2* overexpression. The development of mammary glands in control, p53<sup>-/-</sup>, and BLGMDM2 (TG 3640) transgenic mice were compared at day 10 of lactation. Whole-mount analysis revealed that, whereas both the control and p53<sup>-/-</sup> glands were fully developed (Fig. 4A and B, respectively), the BLGMDM2 gland had fewer alveolar structures that cluster together to form lobules (Fig. 4C). The lack of mammary gland development made it possible to see individual ducts (Fig. 4C), which are normally masked by the alveolar lobules in a fully developed nontransgenic lactating gland (Fig. 4A). Comparison of H&E stained sections from control (Fig. 4D) and p53<sup>-/-</sup> (Fig. 4E) mammary glands at day 10 of lactation showed a similar morphology, that of a single layer of columnar epithelial cells lining the ducts and alveoli. It has been reported previously that p53<sup>-/-</sup> mice develop normally and are able to nurse their pups (Donehower et al. 1992; Li et al. 1996). By comparison, the ductal and alveolar cells of the BLGMDM2 gland (Fig. 4F) were abnormal: Fewer epithelial cells were present in the alveoli, and the cells were typically two to three times larger than the controls. Histological analysis of 500 epithelial cells of the transgenic gland showed that ~28% had enlarged nuclei, 13% were bi- or multinucleate, and 5% were binucleate and enlarged. Occasional mitotic figures were also observed (<1%). All six lines expressing high levels of the BLGMDM2 transgene were evaluated histologically, and similar phenotypes were observed. MDM2 expression was monitored using immunohistochemical analysis with an MDM2-specific polyclonal antiserum. Sections from BLGMDM2 mammary glands (TG3640) showed el-





**Figure 4.** Comparison of mammary glands from normal,  $p53^{-/-}$ , and BLGMDM2 (TG3640) mice at day 10 of lactation. Whole mounts of mammary glands were analyzed from control (A),  $p53^{-/-}$  (B), and BLGMDM2 (C) mice. (A–C) Magnification, 80 $\times$ . H&E stained sections of glands from control (D),  $p53^{-/-}$  (E), and BLGMDM2 (F) mice. Immunohistochemical detection of MDM2 from control (G),  $p53^{-/-}$  (H), and BLGMDM2 (I) mammary sections. (D–I) Magnification, 400 $\times$ .

evated expression of MDM2 in the nuclei of mammary epithelial cells from day 10 of lactation (Fig. 4I) relative to sections from either control or  $p53^{-/-}$  mice (Fig. 4G,H).

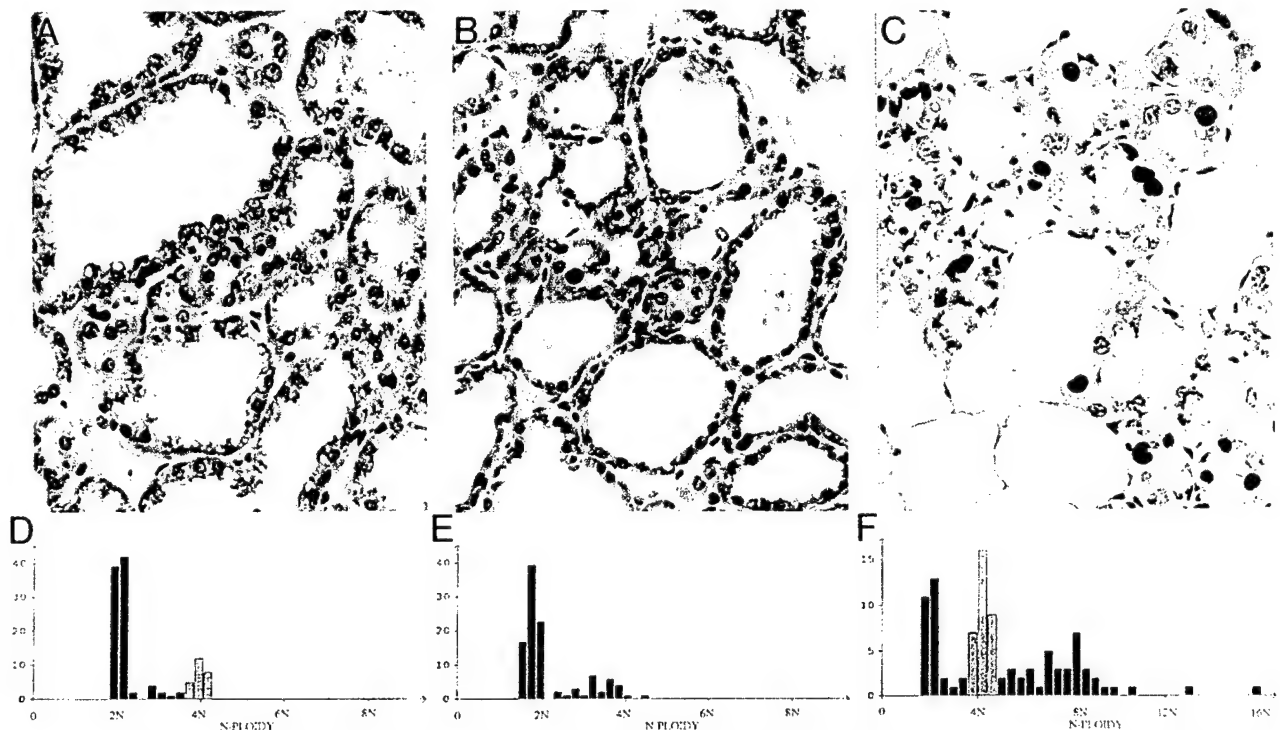
#### *Cell cycle analysis of cells from BLGMDM2 transgenic glands*

Our results demonstrated that MDM2 overexpression during the lactation cycle in the mammary gland of transgenic mice results in a decrease in epithelial cell number and inhibits gland development. To determine whether the decrease in the number of epithelial cells seen in BLGMDM2 transgenic mice was a result of an increased level of apoptosis, an *in situ* terminal deoxynucleotidyl transferase (TdT) assay was performed. Less than 1% of mammary epithelial cells in the control and transgenic mammary glands stained positively for apoptosis at day 10 lactation, demonstrating that apoptosis did not account for the decrease in the number of cells in the BLGMDM2 transgenic gland (data not shown).

The lack of mammary gland development coupled

with the presence of epithelial cells abnormally large and multinucleated led to further studies of the cell cycle in BLGMDM2 mice. To investigate the effect of MDM2 expression on proliferation of mammary epithelial cells, the percentage of cells in S phase was examined in non-transgenic control,  $p53^{-/-}$  and BLGMDM2 TG3640 transgenic mice at day 10 of lactation (Fig. 5A–C) using bromodeoxyuridine (BrdU) incorporation as an index. As expected, few epithelial cells from control and  $p53^{-/-}$  glands, which are terminally differentiated by day 10 of lactation, were synthesizing DNA actively. Less than 1% of the control cells incorporated BrdU (Fig. 5A), whereas ~4% of mammary epithelial cells in the  $p53^{-/-}$  gland incorporated BrdU (Fig. 5B). Surprisingly, 12% and 23% of the mammary epithelial cells from BLGMDM2 glands from two transgenic lines (TG3639 and TG3640, respectively) incorporated BrdU at day 10 of lactation (Fig. 5C; Table 1), indicating these cells are synthesizing DNA actively.

As an alternate method of determining the fraction of cells undergoing DNA synthesis, detection of the proliferating cell nuclear antigen (PCNA) was used to identify



**Figure 5.** Detection of BrdU incorporation and ploidy analysis in epithelial cell nuclei of control, p53<sup>-/-</sup>, and BLGMDM2 (TG3640) transgenic mammary glands. Immunohistochemistry was used to detect BrdU in nuclei of mammary epithelial cells from control (A), p53<sup>-/-</sup> (B), and BLGMDM2 (C). [A–C] Magnification, 400 $\times$ . Histograms of DNA content showing the nuclear ploidy of control (D), p53<sup>-/-</sup> (E), and BLGMDM2 (F) mammary epithelial cells. The y-axis represents cell numbers.

cells in S phase in situ (Foley et al. 1991). PCNA staining analysis was performed in control, p53<sup>-/-</sup>, and two different lines of BLGMDM2 transgenic mice (Table 1). In this study, BrdU incorporation and the results from PCNA staining correlated well. Two glands analyzed from each of two TG3640 and two TG3639 mice showed that the BLGMDM2 S-phase index was four to five times that of the p53<sup>-/-</sup> mice (Table 1).

To investigate the apparent paradox that MDM2 overexpression increased the S-phase index yet did not result

in an increase in the number of epithelial cells, we analyzed cells for ploidy. Ploidy analysis was carried out by quantitating the relative optical density (OD) of Feulgen-stained nuclei (Schiff's reagent) using the DNA Image Cytometry system (LabCorp). The Feulgen reaction product binds stoichiometrically to DNA so that the integrated OD obtained by image cytometry is proportional to the amount of DNA in the nucleus (Schulte and Wittekind 1989). Mammary epithelial cell nuclei from control mice were used to establish 2N and 4N DNA

**Table 1.** Cell cycle analysis of mammary epithelial cells from BLGMDM2 transgenic mice

Mouse	BrdU labeling index <sup>a</sup>	PCNA S-phase labeling index <sup>b</sup>	Ploidy <sup>c</sup>		
			2N	4N	>4N
Control	1% (11/1091)	1% (17/1202)	66% (287/438)	34% (151/438)	0
p53 <sup>-/-</sup>	4% (79/2182) <sup>d</sup>	5% (48/1032) <sup>d</sup>	80% (334/419)	20% (85/419)	0
BLGMDM2					
TG 3639	12% (195/1668)	18% (196/1072)	20% (81/407)	40% (163/407)	40% (163/407)
BLGMDM2					
TG 3640	22% (194/892)	24% (238/1008)	27% (129/471)	39% (186/471)	33% (156/471)

BrdU labeling, PCNA staining, and ploidy analysis were conducted on two mammary glands from each of two control, p53<sup>-/-</sup>, BLGMDM2 TG3639, and BLGMDM2 TG3640 mice.

<sup>a</sup>Percentage of mammary epithelial cells labeled with BrdU after a 1-hr pulse (positive cells/total examined).

<sup>b</sup>Percentage of mammary epithelial cells that stain with anti-PCNA antibody (positive cells/total examined).

<sup>c</sup>Percentage of mammary epithelial cell nuclei with DNA content of 2N, 4N, or >4N (positive cells/total examined).

<sup>d</sup>Analysis conducted on two mammary glands from one mouse.



values. The OD of DNA in p53<sup>-/-</sup> and BLGMDM2 transgenic nuclei was then used to calculate the relative OD. The data showed that nuclei from both control (Fig. 5D) and p53<sup>-/-</sup> (Fig. 5E) epithelial cells were predominantly in G<sub>1</sub> with a DNA content peak at 2N in the fully differentiated day 10 lactation gland. In contrast, BLGMDM2 mammary epithelial nuclei (Fig. 5F) were found to be polyploid, with OD values at 4N, 8N, and 16N, or up to eight copies of a genomic equivalent, relative to the normal DNA content. Ploidy analysis also revealed a population of nuclei with intermediate DNA contents (relative to 4N, 8N), but we could not determine whether the nuclei with intermediate DNA content were synthesizing DNA actively at the time of analysis. Results from DNA image analysis indicated that 35% to 40% of the nuclei from BLGMDM2 mammary epithelial cells were polyploid, suggesting that deregulated expression of MDM2 led to multiple rounds of DNA synthesis without cytokinesis.

#### *The phenotype caused by deregulated expression of MDM2 is independent of p53 function*

To determine the contribution of p53 to glandular abnormalities and polyploidy resulting from MDM2 overexpression, BLGMDM2 mice (TG 3640) were crossed into the p53<sup>-/-</sup> background. PCR analysis was used to genotype the animals used in this study (Fig. 6). Histological samples from the p53<sup>-/-</sup> and BLGMDM2/p53<sup>-/-</sup> mammary glands are shown in Figure 7A and B, respectively. The BLGMDM2/p53<sup>-/-</sup> mammary gland showed a lack of development and fewer lobulo-alveolar structures with enlarged and multinucleated cells (Fig. 7B), very similar to the BLGMDM2 transgenic mammary gland (Fig. 4F). BrdU incorporation studies (Fig. 7C,D) and ploidy analysis (Fig. 7E,F) showed that 18% of the mammary epithelial cells were undergoing DNA synthesis, and 29% of the nuclei were polyploid in BLGMDM2/p53<sup>-/-</sup> mice. These results demonstrate that deregulated

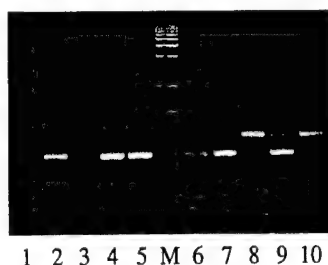
expression of MDM2 in the mammary gland of transgenic mice uncoupled S phase from karyokinesis and/or cytokinesis, independent of p53.

#### *Development of mammary tumors in BLGMDM2 transgenic mice*

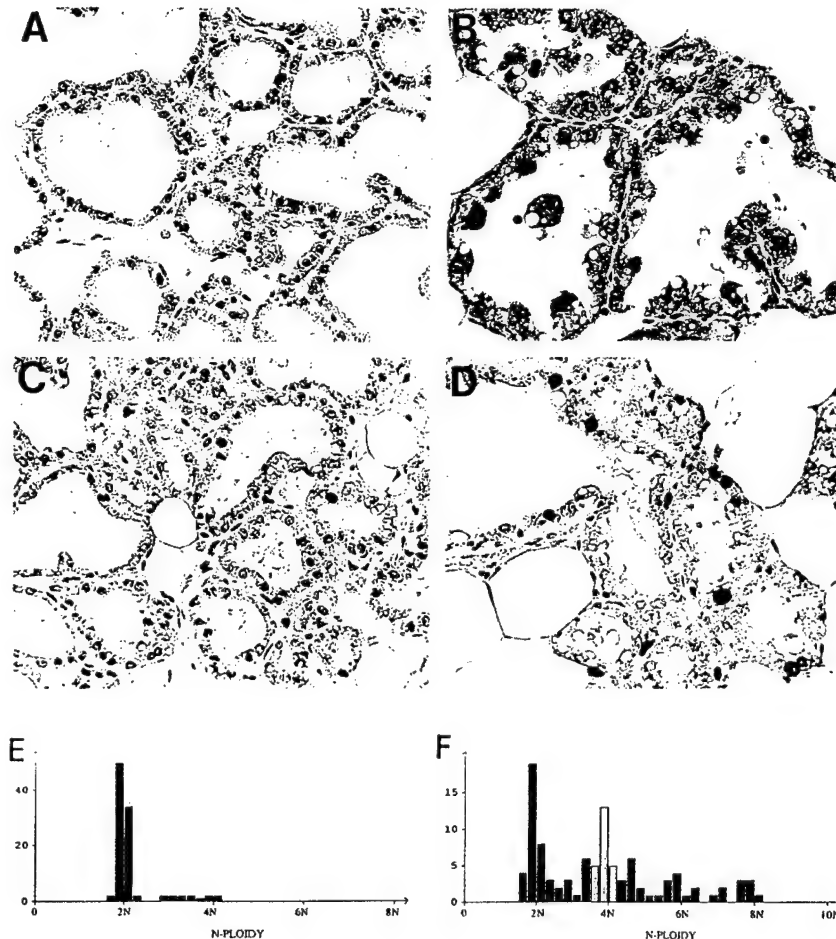
Previous studies have demonstrated *mdm2* can function as an oncogene in vitro and is frequently amplified in human sarcomas (Fakhrazadeh et al. 1991; Oliner et al. 1992; Finlay 1993), suggesting that MDM2 overproduction is a causative factor in tumor formation. Aging studies were performed on retired breeders to ascertain the tumorigenic potential of *mdm2* deregulation. To date, tumors have been observed in 5/31 (16%) retired breeders (lines TG3640, TG3643, and TG3646). These tumors developed in mammary glands between 14 and 18 months of age. In addition, multiple tumors in multiple glands were observed in these mice. No incidence of mammary tumor formation has been observed in non-transgenic or inbred C57BL/6J control mice maintained for up to 18 months in our colonies (data not shown). Figure 8 shows representative histological sections from two of the mammary tumors. Figure 8A shows an adenocarcinoma with characteristic acinar structures adjacent to an adenoacanthoma, which has a mixture of glandular tumor tissue and keratinized epidermoid tissue. An adenocarcinoma from another animal that appeared more ductal in structure is shown in Figure 8B. In addition to the tumors, hyperplasia and characteristic preneoplastic hyperplastic alveolar nodules (HANs) were evident in several glands of aged animals (data not shown). Western blot analysis demonstrated the presence of MDM2 protein in tumors from two BLGMDM2 mice (Fig. 8C). MDM2 expression was undetectable in normal mammary tissue (data not shown). As a control for MDM2 protein, the *mdm2*-amplified 3T3 DM cell line (Fakhrazadeh et al. 1991) was used. Transgenic *mdm2* expression was confirmed using T2 RNase protection analysis (data not shown).

#### Discussion

The present study describes the phenotype of transgenic mice with deregulated expression of MDM2 in the mammary gland during gestation and lactation. Three important points demonstrated by the data presented in this paper need to be emphasized. First, MDM2 overexpression resulted in cell cycle abnormalities and poorly developed mammary glands during gestation and lactation. S-phase analyses showed that transgenic epithelial cells were actively undergoing DNA synthesis, and ploidy analysis demonstrated that the cells had undergone multiple rounds of replication without completing cell division. Second, these effects occurred independently of the presence of p53. Mammary epithelial cells overexpressing MDM2 and null for p53 had a high S-phase index accompanied by an increase in polyploid cells comparable to that observed in a transgenic MDM2 wild-type p53 background. Third, deregulated expression of



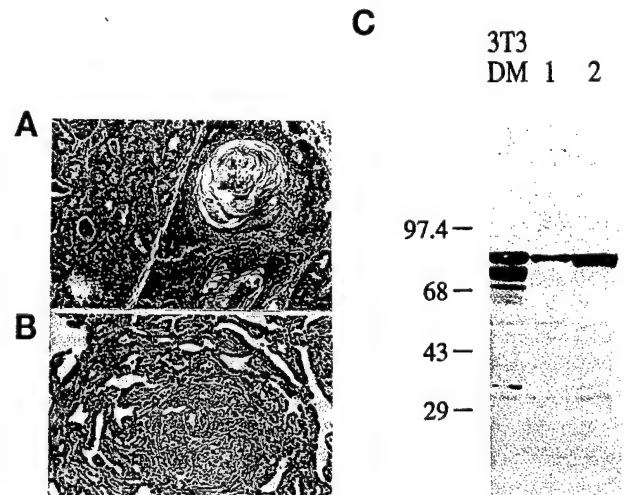
**Figure 6.** Genotyping of p53 homozygous null/BLGMDM2 transgenic animals. (Lanes 1–5) PCR was performed with BLGMDM2 primers. (Lane 1) Nontransgenic wild-type p53; (lane 2) TG3640 BLGMDM2; (lane 3) p53<sup>-/-</sup>; (lane 4) TG3640 BLGMDM2/p53<sup>+/+</sup>; (lane 5) TG3640 BLGMDM2 p53<sup>-/-</sup>. (Lanes 6–10) PCR analysis was performed with p53 primers. (Lane 6) Wild-type p53; (lane 7) TG 3640 BLGMDM2; (lane 8) p53<sup>-/-</sup>; (lane 9) TG3640 BLGMDM2 p53<sup>+/+</sup>; (lane 10) BLGMDM2/p53<sup>-/-</sup>.



**Figure 7.** Comparison of histology and ploidy of mammary epithelial cells from glands of  $p53^{-/-}$  and TG3640 BLGMDM2/ $p53^{-/-}$  mice. H&E staining of sections from  $p53^{-/-}$  (A) and BLGMDM2/ $p53^{-/-}$  (B) mammary glands. Immunohistochemistry was used to detect BrdU incorporation in mammary epithelial cells of  $p53^{-/-}$  (C) and BLGMDM2/ $p53^{-/-}$  (D) mice. (A-D) Magnification, 400 $\times$ . Histograms of DNA content showing ploidy of  $p53^{-/-}$  (E) and BLGMDM2/ $p53^{-/-}$  (F) mammary epithelial cells. The y-axis represents cell numbers.

MDM2 can result in mammary tumor development following a long latency. Tumors have been observed in ~16% of animals by 14–18 months of age.

The deregulated expression of MDM2 in the mammary gland during gestation and lactation resulted in a loss of coordination between DNA synthesis and mitosis; however, the mechanisms underlying this phenotype have not been defined. MDM2 overproduction may induce the initiation (and reinitiation) of S phase, prevent S-phase completion, or inhibit entry into mitosis. Given what is known about MDM2 function, the hypothesis we favor most is that MDM2 overexpression can constitutively provide signals that repeatedly induce the initiation of DNA synthesis, but cannot induce all of the cellular pathways necessary for an orderly transition into  $G_2$  and M. This hypothesis is supported by the fact that MDM2 can inhibit the activity of the p53 and the Rb tumor suppressors (Momand et al. 1992; Chen et al. 1993, 1995; Oliner et al. 1993; Xiao et al. 1995), and can stimulate the activity of the E2F1 transcriptional activator (Martin et al. 1995). E2F1 induces expression of a variety of genes necessary for DNA synthesis, such as DNA polymerase  $\alpha$ , thymidylate synthase, PCNA, ribonucleotide reductase, thymidine kinase, and dihydrofolate reductase (DeGregori et al. 1995). Thus, one possible



**Figure 8.** Histological and Western blot analysis of mammary tumors from BLGMDM2 transgenic mice. (A) A carcinoma (left) was found adjacent to an adenocanthoma (right) in mammary gland 3L from founder TG3643. (B) An adenocarcinoma from a first-generation TG 3643 mouse. (A,B) Magnification, 200 $\times$ . (C) Western blot analysis to detect MDM2 in tumors of transgenic mice. (Lane 1) 3T3 DM cell lysate; (lane 2) tumor lysate from TG3643 founder; (lane 3) tumor lysate from TG3646 founder.

mechanism that can explain this transgenic phenotype is that overexpression of MDM2 constitutively augments E2F1, an activity that keeps the cells in S phase. Numerous studies have demonstrated, however, that inappropriate expression of E2F1 results in the initiation of S phase followed by p53-mediated apoptosis (Qin et al. 1994; Shan and Lee 1994; Wu and Levine 1994; Hiebert et al. 1995). The lack of apoptosis observed in our system was most likely attributable to the ability of MDM2 to also inhibit p53 function. We are currently testing this hypothesis.

Another important observation made in our study is that deregulated expression of *mdm2* leads to tumor development. Approximately 16% of transgenic mice overexpressing MDM2 developed mammary adenocarcinomas and adenocanthomas following a latency period of 14–18 months. These data provide direct evidence in vivo that *mdm2* deregulation causes tumor formation. In the BLGMDM2 mammary glands, MDM2 expression decreased in most of the epithelial cells after weaning and the cells were cleared through involution. However, the small percentage of mammary epithelial cells that persisted through each cycle (some of which may have been polyploid) were perhaps genetically unstable. Secondary mutations could accumulate that would allow these epithelial cells to retain deregulated expression of MDM2 and to overcome the uncoupling of S phase from mitosis and cycle normally. A critical role for MDM2 in this system could be inhibition of wild-type p53 function leading to tumor formation.

This collection of cellular and glandular abnormalities seen in the BLGMDM2 transgenic mice appears to be unique compared with other transgenic mice expressing oncogenes in the mammary gland (Cardiff and Munn 1995). Some aspects of the phenotypes we observed, for example, lactation difficulties and adenocarcinomas with varying latency, have been observed in transgenic mice overexpressing the *neu*, *Wnt-1*, or cyclin D1 oncogenes (Muller et al. 1988; Tsukamoto et al. 1988; Wang et al. 1994). However, poor glandular development as seen in the BLGMDM2 transgenics contrasts the glandular hyperplasia seen in these other mice. In addition, our studies have addressed changes in ploidy and multiple rounds of DNA synthesis in the abnormal mammary epithelial cells.

Previous results had suggested that *mdm2* amplification would have the same effect as mutational inactivation of p53, because MDM2 can bind to and inactivate p53. The results of this study demonstrate for the first time that MDM2 has a role independent of inhibition of p53 function. In one study of human sarcomas, the immunohistochemical detection of both mutant p53 and MDM2 proteins in the same tumors correlated with poor survival of the patients (Cordon-Cardo et al. 1994). Taken together, these findings imply that MDM2 may play a larger role in cellular transformation than simply inactivating p53. Although the shortened lifespan of the p53 null mouse (Donehower 1992; Jacks et al. 1994) prevents an analysis of tumor formation in the MDM2 transgenic in the absence of p53 function, experiments

are under way to determine whether overproduction of MDM2 and p53 mutant protein increases the tumor rate in transgenic mice.

In conclusion, deregulated expression of MDM2 in the mammary gland of transgenic mice uncouples S phase and mitosis, leading to cellular defects including polyploidy. This phenotype ultimately leads to mammary tumorigenesis. In humans, the vast majority of the documented cases of *mdm2* amplification/overexpression are found in human sarcomas, a tumor derived from mesenchymal lineage. Our data suggest that MDM2 may play a role in tumors of other cell lineages as well.

## Materials and methods

### Generation of transgenic mice

The ovine  $\beta$ -lactoglobulin promoter expression vector was a gift from A.J. Clark (Edinburgh Research Station, Scotland, UK). An *mdm2* minigene bearing two introns was constructed by replacement of the *AatII*-*EcoO109I* cDNA fragment with the corresponding fragment (1400 bp) from the *mdm2* minigene containing introns 7 and 8 (Montes de Oca Luna et al. 1996). An *EcoRV* fragment containing the *mdm2* minigene was subcloned into the *EcoRV* site of the  $\beta$ -lactoglobulin expression vector (Harris et al. 1991; Bortner and Rosenberg 1995). Digestion with *SalI* and *XbaI* released the 9.7-kb transgene from the vector. The fragment was purified on a 0.8% low melting agarose gel by cutting out the transgenic fragment and digesting with *GELase* (Epicentre). The DNA was then purified by Resource Q anion exchange FPLC (Pharmacia) and ethanol precipitated. The fragment was resuspended in 10 mM Tris (pH 7.4), 0.1 mM EDTA at a final concentration of 3 ng/ $\mu$ l. The construct was coinjected with a dominant coat color marker (Kucera et al. 1996). Microinjection of B6C3F2 zygotes was performed as described previously (Osborn et al. 1987). Mice with the p53 null allele were obtained from Tyler Jacks (Jacks et al. 1994) and backcrossed onto C57Bl/6J.

### PCR analysis and Southern blotting

DNA was isolated from 1-cm tail biopsies and subjected to PCR analysis. Two different primer sets were used to identify transgenic animals. One set spans the BLG promoter/MDM2 transgene junction. The forward primer is in the BLG promoter (BLG 5'-CCTGTCCTTGCTAAGAGGCTGAC-3'), and the reverse primer is in the *mdm2* gene (MDM2 5'-CGAAGCTGGAATC-TGTGAGGTG-3') and yields a PCR fragment of 590 bases. The second set of primers amplifies a fragment from the BLG promoter (F2 5'-CCGTCCTGGGGTTATTGACTC-3'; B2 5'-TCCAAACCATGTGACCGTGG-3') and yields a predicted fragment of 200 bases. In genotyping the p53<sup>-/-</sup> animals, primers X6.5 (5'-ACAGCGTGGTGGTACCTTAT-3') and X7 (5'-TATACTCAGAGCCGGCCT-3') gave a 375-bp band for the wild-type allele and MCI (5'-ATCAGGACATAGCGTTGGC-TACCC-3') and X7 gave a 525-bp band for the p53<sup>-/-</sup> allele (Jacks et al. 1994). Both bands were visible in p53<sup>+/+</sup> animals. PCR assay conditions were 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 10  $\mu$ M of deoxynucleotide triphosphate mixture, 0.65 ng/ $\mu$ l of primers, 3  $\mu$ l of 1:100 dilution of tail DNA, and 0.5 units of native *Taq* DNA polymerase (Perkin Elmer-Cetus) in a 50- $\mu$ l reaction mixture. Forty cycles of denaturation (1 min, 94°C), annealing (1 min, 52°C), and extension (2 min, 72°C) were performed in a Techne PHC-3 thermal cycler. Founder animals that were positive in the PCR assay were con-

firmed by Southern blotting analysis. Approximately 10 µg of genomic tail DNA was digested with *Bam*HI and electrophoresed on a 0.8% agarose gel. The DNA was transferred to a nitrocellulose membrane using standard procedures (Sambrook et al. 1989) and hybridized with a radiolabeled 1.5-kb *Bam*HI-*Sph*I fragment from the BLG promoter, yielding a band at 2.2 kb that is specific for the transgene.

#### Preparation and analysis of RNA

Total cellular RNA was prepared from mouse tissues using Tri Reagent (Molecular Research Center, Inc.) according to the manufacturer's instructions. RNA expression was analyzed in a T2 RNase protection assay. To make the MDM2 antisense probe the *Bam*HI fragment of the *mdm2* minigene was subcloned into Bluescript KS<sup>+</sup> (Stratagene). The plasmid was linearized at the *Sal*I site and used as a template for in vitro transcription with T7 RNA polymerase (Boehringer Mannheim). For an internal control, a 300-nucleotide antisense riboprobe for the  $\beta$ -actin gene (Ambion) was synthesized. Twenty-five to fifty micrograms of total cellular RNA was combined with  $5 \times 10^5$  to  $1 \times 10^6$  cpm of synthesized probe and ethanol-precipitated and dried. The pellet was resuspended in 40 µl of hybridization buffer containing 80% formamide, 40 mM 1,4 piperazinediethanesulfonic acid (PIPES, pH 6.7), 0.4 M NaCl, and 1 mM EDTA and incubated overnight at 60°C. Three hundred and fifty microliters of ice-cold T2 RNase digestion buffer [50 mM sodium acetate (pH 4.5), 2 mM EDTA, 100 mM NaCl] containing 20 units/ml of T2 RNase were added to each sample and incubated at 33°C for 1 hr. Samples were ethanol-precipitated, resuspended in 8 µl of gel loading buffer (Ambion), subjected to electrophoresis through a 5% polyacrylamide gel, and analyzed by autoradiography.

#### Histology and immunohistochemistry

Tissues obtained from animal necropsies or mammary gland biopsies were fixed in Z-fix neutral buffered formalin (Anatech) or Bouin's fixative (Sigma). Five-micrometer thick paraffin sections were stained with H&E for histological analysis. Mice received an intraperitoneal injection of BrdU (Boehringer-Mannheim) at 120 mg per kg body weight 1–1.5 hr before sacrifice to label cells in S phase (Kim et al. 1993). For BrdU, PCNA, and MDM2 staining, 5-µm thick sections were placed on ProbeOn<sup>+</sup> slides (Fisher Scientific) to insure adhesion during processing. Tissue sections were incubated with a monoclonal antibody to BrdU (1:500, Accurate Chemical and Scientific Corporation) or PCNA (1:500, Dako Corporation). Rabbit polyclonal antiserum specific for the MDM2 protein was a generous gift from Arnold Levine (Princeton University). The MDM2 antiserum was diluted 1:250 for immunocytochemistry. The primary antibodies were detected using the Vectastain ABC immunoperoxidase system with 3,3'-diaminobenzidine tetrahydrochloride (DAB) as the chromagen substrate. In addition, the DAB enhancer (Zymed) was used for MDM2 detection. Positive staining for PCNA was determined based on cellular distribution and intensity of the brown to black staining, which correlates with the different phases of the cell cycle as reported previously (Eldridge et al. 1990, 1993; Foley et al. 1991). For whole-mount analysis, the first abdominal (4th) mammary gland was dissected and stretched on a glass slide. After air drying, the gland was defatted and fixed overnight in acetone. The glands were then stained in Harris' hematoxylin overnight, destained in a solution of 2% HCl in 70% ethanol, and dehydrated in a graded ethanol series. The dehydrated tissue was cleared overnight in toluene and mounted.

#### Ploidy analysis

Paraffin sections were cut 6 µm thick and placed on Probe On<sup>+</sup> slides. Tissue sections were stained using the Feulgen staining kit (Becton Dickinson Cellular Imaging systems). An unstained calibration slide and an unstained mammary sample were included as method controls in the staining procedure. Nuclei in sections from nontransgenic control mammary epithelial cells were used to establish the 2N DNA content using DNA Image Cytometry (Roche Pathology WorkStation, Roche Image Analysis Systems, Inc., now LabCorp). The optical density of nuclei from transgenic mice was quantitated relative to the control nuclei (Schulte and Wittekind 1989; Cohen 1996; Marchevsky et al. 1996). The OD of ~100 nuclei per sample was determined, and the results were graphed. The prominent histogram peaks were labeled to give an overall DNA index for each peak (LabCorp, Research Triangle Park, NC).

#### Western blot analysis

At necropsy, tissues were frozen at -70°C. For Western analysis, lysates of tissues were made in lysis buffer (50 mM Tris at pH 7.4, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate) containing protease inhibitors by dounce homogenization followed by sonication. For an MDM2 control, 3T3 DM cell (Fakhrazadeh et al. 1991) pellets were lysed in 50 mM Tris at pH 8, 5 mM EDTA, 150 mM NaCl, and 0.5% NP40 containing protease inhibitors. After centrifugation, a protein assay (Biorad) was used to determine the protein concentration of lysates. Fifty micrograms of protein lysate were loaded on a 4%–12% NuPAGE gradient gel (Novex) and electrophoresed at 200 V. Proteins were transferred to nitrocellulose using the NuPAGE (Novex) transfer system according to the manufacturer's instructions. Blots were blocked with 2.5% milk in TBS-T for 1 hr and then incubated with an MDM2-specific polyclonal antiserum at 1:500 dilution for 1 hr at room temperature. After being washed in five changes of TBS-T, the blot was incubated with a Donkey anti-rabbit antibody (Amersham) at a 1:4000 dilution for 1 hr at room temperature. The MDM2 protein was detected using the ECL detection system (Amersham).

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